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Abstract: Public goods cooperation is common in microbes, and there is much interest in understanding how such traits evolve. Research in recent years has identified several important factors that shape the evolutionary dynamics of such systems, yet few studies have investigated scenarios involving interactions between multiple public goods. Here, we offer general predictions about the evolutionary trajectories of two public goods traits having positive, negative or neutral regulatory influence on one another's expression, and we report on a test of some of our predictions in the context of Pseudomonas aeruginosa's production of two interlinked iron-scavenging siderophores. First, we confirmed that both pyoverdine and pyochelin siderophores do operate as public goods under appropriate environmental conditions. We then tracked their production in lines experimentally evolved under different iron-limitation regimes known to favour different siderophore expression profiles. Under strong iron limitation, where pyoverdine represses pyochelin, we saw a decline in pyoverdine and a concomitant increase in pyochelin – consistent with expansion of pyoverdine-defective cheats derepressed for pyochelin. Under moderate iron limitation, pyochelin declined – again consistent with an expected cheat invasion scenario – but there was no concomitant shift in pyoverdine because cross-suppression between the traits is unidirectional only. Alternating exposure to strong and moderate iron limitation caused qualitatively similar though lesser shifts compared to the constant environment regimes. Our results confirm that the regulatory interconnections between public goods traits can significantly modulate the course of evolution, yet also suggest how we can start to predict the impacts such complexities will have on phenotypic divergence and community stability.

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Evolutionary dynamics of interlinked public goods traits: an experimental study of siderophore production in *Pseudomonas aeruginosa*

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Abstract

Public goods cooperation is common in microbes, and there is much interest in understanding how such traits evolve. Research in recent years has identified several important factors that shape the evolutionary dynamics of such systems, yet few studies have investigated scenarios involving interactions between multiple public goods. Here we offer general predictions about the evolutionary trajectories of two public goods traits having positive, negative or neutral regulatory influence on one another's expression, and we report on a test of some of our predictions in the context of *Pseudomonas aeruginosa*'s production of two interlinked iron-scavenging siderophores. First, we confirmed that both pyoverdine and pyochelin siderophores do operate as public goods under appropriate environmental conditions. We then tracked their production in lines experimentally evolved under different iron-limitation regimes known to favour different siderophore expression profiles. Under strong iron limitation, where pyoverdine represses pyochelin, we saw a decline in pyoverdine and a concomitant increase in pyochelin – consistent with expansion of pyoverdine-defective cheats derepressed for pyochelin. Under moderate iron limitation, pyochelin declined – again consistent with an expected cheat invasion scenario – but there was no concomitant shift in pyoverdine because cross-suppression between the traits is unidirectional only. Alternating exposure to strong and moderate iron-limitation caused qualitatively similar though lesser shifts compared to the constant-environment regimes. Our results confirm that the regulatory inter-connections between public goods traits can significantly modulate the course of evolution, yet also suggest how we can start to predict the impacts such complexities will have on phenotypic divergence and community stability.

Introduction

Bacteria frequently cooperate by sharing secreted secondary metabolites (West et al., 2007). These shared products, though costly for the individual to produce, can benefit other individuals, or the bacterial collective in general and, in such cases, they constitute public goods (West et al., 2007). Bacterial public goods include structural materials for building biofilms (Nadell et al., 2009), signalling molecules for communication (Williams, 2007), enzymes to digest food (Diggle et al., 2007), biosurfactants for group motility (Kearns, 2010), toxins to fight competitors (Jousset, 2012), and chelating agents to scavenge essential metals (Griffin et al., 2004). Such cooperation can generate enormous group-level benefits, yet it is also famously vulnerable to being undermined by "cheat" variants that contribute little or nothing to the collective stock of public goods, and thereby escape their fair share of costs while still benefitting from the cooperative efforts of others (West et al., 2006). By exploiting cooperators, cheats can increase in frequency – even if this ultimately harms the collective as a whole. However, as numerous studies have revealed, this "social dilemma" can be modulated by a range of mechanisms that constrain cheat fitness and thereby maintain cooperation (e.g. Ross-Gillespie et al., 2009, Brockhurst et al., 2010, Kümmerli & Brown, 2010, Xavier et al., 2011, Dandekar et al., 2012, Drescher et al., 2014). For example, limited dispersal in viscous environments can both limit cheats' access to co-operators and their precious public goods (Kümmerli et al., 2009a; Julou et al., 2013). Also, cheats can lose their relative advantage as they become more common (negative frequency-dependent payoffs; Ross-Gillespie et al., 2007, Jousset et al., 2009, Raymond et al., 2012).

Although this body of work has greatly aided our understanding of the dynamics of public goods traits, the scenarios investigated in these studies remain, by and large, simplified approximations of what really goes on in natural microbial communities. In particular, most studies consider just one model trait at a time. Under natural conditions, however, bacteria have to juggle a portfolio of various different public goods, and hence, they could routinely find themselves simultaneously participating in multiple public good dilemmas on multiple fronts (Brown & Taylor, 2010; Mellbye & Schuster, 2014).

Another important complication is that the production of different public goods is often linked, positively or negatively, at the regulatory level (Nadal Jimenez et al., 2012). Consequently, selection for or against one public good might have pleiotropic consequences for other public goods (Sandoz et al., 2007, Harrison & Buckling, 2009, Driscoll et al., 2011, Inglis et al., 2012, Jousset et al., 2013, Friman et al., 2013). Ultimately, if we want to understand the evolutionary dynamics of social traits in complex natural microbial communities, we will need to consider also the joint effects of superimposed public goods dilemmas, and the nature of the links between them (Brown & Taylor, 2010).

Here, we take on this issue. We start by developing general predictions for any two-trait case, where traits, that may or may not be public goods, are linked positively, negatively, or not at all (Fig. 1a). Next, we test a subset of our predictions in a specific empirical test case, tracking expression of two regulatorily-linked public goods in the bacterium *Pseudomonas aeruginosa* during experimental evolution. *P. aeruginosa*, an opportunistic human pathogen, produces and secretes two siderophores to scavenge iron. The first, pyoverdine, is a more effective iron-chelator but is metabolically more costly to produce, while the second, pyochelin, is less effective but cheaper (Cornelis, 2010). Importantly, both pyoverdine and pyochelin are freely diffusing exoproducts, and, as we show below, both can function as public goods under suitable conditions. The relative investment into one or the other siderophore is dependent on relative iron availability: bacteria up-regulate pyoverdine and repress pyochelin when iron limitation is strong, but as it weakens, they reduce pyoverdine production and switch instead to pyochelin (Dumas et al., 2013). Mechanistically, this switch is mediated by (a) direct (albeit incomplete) suppression of pyochelin synthesis by pyoverdine; and (b) a strong negative feedback (operating via the ferric uptake regulator, Fur) that shuts down pyoverdine synthesis whenever iron is more readily available (Dumas et al., 2013).

Given these biological details, we can derive qualitative predictions for the evolutionary trajectories of pyoverdine and pyochelin production under different types of iron stress (Fig. 1b). Under strong iron

limitation, where *P. aeruginosa* cells produce mainly pyoverdine, we expect cheats, defective for this siderophore to emerge and spread (Fig. 1b, upper right block). As a consequence of their lowered pyoverdine production, we then expect the pleiotropic derepression (i.e. increase) of pyochelin production in these mutants – turning pyoverdine-cheats into pyochelin-cooperators. Thus exposed to selection, pyochelin too should then decline, since pyochelin-defective cheats now also should emerge and spread. Double mutants, defective for production of either siderophore but still able to exploit both, could have the greatest relative advantage. Under more moderate iron limitation, meanwhile, where bacteria mainly produce pyochelin, we expect mutants defective for pyochelin production to emerge and to drag down the aggregate expression of pyochelin (Fig. 1b, lower right block). Unlike the scenario with strong iron limitation, however, we do not expect compensatory upregulation of the other siderophore, because the regulatory link is strictly unidirectional – pyoverdine influences pyochelin production, but not the other way round (Dumas et al., 2013). Finally, in a fluctuating environment where iron is alternately strongly- then moderately limited, we would expect selection for both pyoverdine and pyochelin cheats to be weaker, leading to slower and/or less pronounced shifts in the siderophore profiles of the population. This is because mutants defective for one or the other siderophore should have a selective advantage only part of the time. We tested these predictions by tracking siderophore production profiles in replicate populations of the *P. aeruginosa* PAO1 wildtype strain over approximately 300 generations (48 serial transfers) under strong, moderate, and alternating iron-limitation regimes.

Methods

Competition assays to verify cooperator-cheat dynamics

First, we tested our assertion that pyochelin and pyoverdine can function as public goods, exploitable by a non-producing strain. Specifically, we competed strains that can either only produce pyochelin (PAO1Δ*pvdD*, a knockout mutant defective for pyoverdine) or pyoverdine (PAO1Δ*pchEF*, a knockout mutant defective for pyochelin) against a strain that is defective for both siderophores (PAO1Δ*pvdD*Δ*pchEF*), but still possesses the receptors for uptake. All mutants were derived from the

clinical isolate PAO1 (ATCC 15692; Ghysels et al., 2004). PAO1Δ*pvdD* and PAO1Δ*pchEF* were both additionally modified to constitutively-express GFP fluorescence (chromosomal insertion *att*Tn7::Ptac-gfp; Lambertsen et al., 2004), so that we could discriminate between them and the untagged PAO1Δ*pvdD*Δ*pchEF* during competitions.

Starting from freezer stocks, we grew all strains for 24 hours in 10 mL lysogeny broth (LB) cultures (37°C, 220 r.p.m), then standardised optical densities, diluting the denser cultures with 0.8% NaCl so that all strains had similar absorbance at 600 nm. From inocula of ~5×10^5 cells, we then set up new cultures, including monocultures and pairwise mixed cultures (1:4 ratios), in 24-well plates, where each well contained a total of 1.5 mL of iron-limited CAA medium (5 g/L casamino acids, 1.18 g/L $K_2HPO_4*3H_2O$, 0.25 g/L $MgSO_4*7H_2O$, and 25 mM HEPES buffer; all ingredients from Sigma-Aldrich, Switzerland). In the case of PAO1Δ*pchEF* vs. PAO1Δ*pvdDpchEF* (i.e. testing whether pyoverdine is an exploitable public good), we imposed strong iron limitation by supplementing the CAA medium with 100 µg/ml of the human iron chelator apo-transferrin and 20 mM $NaHCO_3$ (required as a co-factor; both ingredients from Sigma-Aldrich, Switzerland). For PAO1Δ*pvdD* vs. PAO1Δ*pvdDpchEF* (i.e. testing whether pyochelin is an exploitable public good), we imposed more moderate iron limitation, by supplementing with 40 mM $NaHCO_3$ only. $NaHCO_3$ itself acts as a mildly effective iron-chelator (Matinaho et al., 2005, Dumas et al., 2013). It is important to note that by adding iron chelators of different strengths, we only manipulated the relative iron availability. The absolute iron-content of the CAA media remained constant, at approximately 1.5 µM (Kümmerli & Ross-Gillespie, 2014).

To track growth under the experimental conditions and – in the case of mixed cultures – any changes in the relative proportion of one strain type to another, we compared samples taken from the starting inocula with samples taken from the mature cultures (after 24 hours incubation at 37°C). We diluted and plated out these samples to LB agar (supplemented with 100 µM $FeCl_3$), and counted the colony-forming units (CFUs) that grew on the plates after 24 hours incubation at 37°C. CFUs of the two

colony types (GFP- vs. non-GFP expressing colonies) were distinguished by photographing plates under brightfield illumination and then again under illumination where only GFP-expressing colonies were visible. By contrasting final proportions with the starting proportions, we calculated relative fitness measures (*sensu* Ross-Gillespie *et al.,* 2007) for the siderophore defective strain PAO1Δ*pvdDpchEF* when in competition against, respectively, the pyoverdine producing strain (PAO1Δ*pchEF*) and the pyochelin producing strain (PAO1Δ*pvdD*), assaying under both high and moderate iron limitation. Analyses of these and other data were performed using R v3.1.1 (http://www.R-project.org/).

Experimental evolution

We evolved eight replicate lines of the PAO1 wildtype in each of three different selective regimes. In the first regime, bacteria faced strong iron limitation (CAA + 100 μg/ml transferrin + 20 mM $NaHCO_3$; see details above), while in the second, iron was only moderately limited (CAA + 40 mM $NaHCO_3$). In the third regime, environmental conditions alternated from transfer to transfer between strong and moderate iron limitation, with four replicates beginning with 'strong' iron limitation conditions and the other four beginning with 'moderate' iron limitation. Experimental evolution was carried out in 24-well plates in a total volume of 1.5 ml medium. Each experimental growth cycle included the incubation of the cultures for 24 hours at 37 °C in a static incubator, during which approximately 6 cell divisions occurred (Dumas & Kümmerli, 2012). Subsequently, we measured the optical properties of all cultures using a multimode plate reader (Tecan Infinite M-200PRO, Tecan Group Ltd., Switzerland). Specifically, we measured the optical density (OD at 600 nm) and the investment into pyoverdine and pyochelin. Both siderophores are naturally fluorescent, so we measured their levels in relative fluorescence units (RFU; where excitation | emission wavelengths were taken as 400|460 nm for pyoverdine and 350|430 nm for pyochelin), and then converted these to *per capita* measures by dividing by OD at 600 nm. To account for the fact that pyoverdine fluoresces more strongly than pyochelin – which results in a considerable signal bleedthrough from the pyoverdine channel into the pyochelin channel – we applied a post-hoc correction procedure described

in Dumas *et al.* (2013). Next, we transferred 15 µl of each culture to fresh medium (i.e. corresponding to a 100-fold dilution) to initiate the next growth cycle. We repeated this procedure for 48 consecutive transfers, resulting in approximately 300 generations of bacterial evolution. Following each transfer, we mixed 500 µl of bacterial cultures with 500 µl LB-glycerol (50% glycerol) for long-term storage at -80°C.

Siderophore production of evolved populations and clones

To assess the evolutionary trajectories of siderophore production levels and growth, we measured cell density, pyoverdine and pyochelin production of 960 clones isolated across different time points during evolution and compared it to the ancestral wildtype. From the frozen record, we plated bacteria from all 24 replicates and from four different time points (12, 24, 36, 48 transfers) onto LB agar. Following overnight incubation at 37 °C, we randomly picked 10 colonies (yielding a total of 960 colonies across replicates and time points), and inoculated them individually into 200 µl LB on a 96-well plate. This step was implemented to ensure that all clones reach comparable optical densities (mean ± SE = 1.131±0.005; OD at 600 nm). Next, we diluted the cultures (final dilution: 10^{-4}), transferred aliquots to both strongly and moderately iron-limited CAA medium, and cultured them under the same conditions as experienced during the evolution experiment (see above). After 24 hours, we measured the optical density of all clones and their investment into pyoverdine and pyochelin – as per methods described above. In addition to this clonal-level assay, we also measured the density and siderophore expression profiles of the evolved populations as a whole. Here, aliquots of the frozen cultures were inoculated with three-fold replication directly to LB, without the plating-and-picking step.

Results

Pyoverdine and pyochelin both function as public goods under appropriate conditions

Our competition assays confirmed that both of *P. aeruginosa*'s siderophores function as beneficial public goods that can be exploited by cheats in a strongly iron-limited environment. Under more

moderate iron stress, however, only pyochelin shows the characteristics of a public goods trait, whereas pyoverdine seems not to be required in this environment (Fig. 2 summarises the competitive performance of non-producers against producers under different levels of iron-limitation; Fig. S1a contrasts the growth performance of all strains when grown as monocultures vs. in mixtures, and Fig. S1b compares per-producer siderophore production effort under mono- vs. mixed cultures). In co-culture under strongly iron-limited conditions, the siderophore non-producer PAO1Δ*pvdD*Δ*pchEF* significantly outperformed the pyoverdine-only-producer PAO1Δ*pchEF*, but also outperformed a pyochelin-only-producer PAO1Δ*pvdD*, indicating that cheats could invade either phenotype under these conditions (LMs with H_0 that relative fitness = 1: $t_{40} = 13.83$, $P < 0.001$ and $t_{40} = 9.60$, $P < 0.001$, respectively). Under moderate iron limitation, meanwhile, the non-producer outperformed only the pyochelin producer ($t_{40} = 3.27$, $P = 0.002$) but not the pyoverdine producer ($t_{40} = -0.45$, $P = 0.657$), indicating that only pyochelin is exploitable in this environment. Comparison of relative fitness values calculated using an alternative approach based on ratios of doublings (as per Lenski et al., 1991) yielded qualitatively similar results (Repeat of LM tests as ordered above: $t_{40} = 13.55$, $P < 0.001$; $t_{40} = 12.36$, $P < 0.001$; $t_{40} = 3.81$, $P < 0.001$; $t_{40} = -0.90$, $P = 0.373$).

Changes in siderophore production during experimental evolution

Isolates from lines evolved under strong iron limitation and assayed under the same conditions showed a decrease in *per capita* pyoverdine investment over time (Fig. 3a; LM: slope = -460.4, $t_{38} = -3.047$, $p = 0.004$). Concomitantly, we saw an increase in pyochelin (LM: slope = 26.584, $t_{38} = 2.922$, $p = 0.005$). In lines evolved under moderate iron limitation, pyoverdine investment also significant declined over time (LM: slope = -95.36, $t_{38} = -2.733$, $p = 0.009$), although, given that the ancestor already showed low expression under these conditions, the absolute magnitude of the shift was relatively minor in this case. Pyochelin, meanwhile, showed a marked decrease under these conditions (LM: slope = -15.846, $t_{38} = -5.671$, $P < 0.001$). Lines evolved under alternating iron-limitation showed the same overall trends, but for the most part, the slopes here were non-significantly different from zero – i.e. towards less pyoverdine (slope = -274.8, $t_{38} = -1.779$, $P = 0.083$) and more pyochelin (slope

= 12.397, t_{38} = 1.493, P = 0.144) under strong iron limitation, and less pyochelin (slope = -7.194, t_{38} = -1.316, P = 0.196) and less pyoverdine (slope = -135.63, t_{38} = -4.501, $P < 0.001$) under moderate iron limitation.

Assaying at a whole-population level (as opposed to averaging across a random sample of isolates, as above), yielded qualitatively similar patterns overall (Fig. 3a, dotted lines) with one notable difference: under either strong or alternating iron limitation, clonal sampling suggested a roughly linear decrease in pyoverdine over time, whereas at a population level, pyoverdine investment first increased before later declining (ANOVA comparison of linear vs. negative quadratic fits: $F_{1,37}$ = 5.286, P = 0.027; $F_{2,36}$ = 7.662, P = 0.008 for the strong- and alternating Fe-limitation regimes respectively). No such difference was observed for pyochelin production patterns, in any of the conditions tested.

Tracking temporal changes in siderophore production as a 'walk' in 2-dimensional phenotype space (Fig. 3b), we saw patterns broadly consistent with those predicted (Fig. 1). Under strong iron limitation, populations shifted from high-pyoverdine / low-pyochelin production towards low-pyoverdine / high-pyochelin production, although we did not see the final phase of the predicted trajectory (i.e towards low-pyoverdine / low-pyochelin production). Under moderate iron limitation, we observed, as predicted, a shift from high to low pyochelin production. Finally, under alternating iron limitations, we observed relatively smaller mean shifts in siderophore production profiles.

Inspection of individual isolates' phenotypes (Fig. 4) revealed relatively few 'extreme' phenotypes – i.e. those showing 'maximal' investment in one siderophore and complete suppression of the other. Moreover, no isolates were observed that were fully defective for both siderophores. Rather, the vast majority of isolates showed only partially reduced siderophore investment, and thus continued to express both siderophores, though typically with altered relative investment in one vs. the other. As compared to lines selected under moderate iron limitation, those subjected to strong iron limitation

(Fig. 4, left column) appear to have diversified into a broader set of phenotypes, with some degree of clustering.

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Discussion

In this study we have developed a conceptual framework to predict how the regulatory links between different social traits could influence their evolutionary trajectories in microbial populations. To test our ideas, we have investigated the evolutionary dynamics of two of *P. aeruginosa*'s public goods
265 traits – the iron-scavenging molecules pyoverdine and pyochelin – in situations where the traits are either hierarchically linked (strong iron limitation, where pyoverdine suppresses pyochelin), or unlinked (moderate iron limitation, where pyochelin is not suppressed). Under the latter scenario, we observed selection against pyochelin (Fig. 3, middle column), which is consistent with our expectation that pyochelin producers would be exploited by cheats under these conditions (Fig. 2). However, we
270 also saw some (weak) selection against pyoverdine. This pattern, we posit, has arisen because pyoverdine provides negligible benefit under these conditions, yet still imposes net costs owing to maintenance of the molecular machinery and baseline expression (Zhang & Rainey, 2013; Kümmerli & Ross-Gillespie, 2014). Under strong iron limitation, where regulatory links between the siderophores are in effect, the evolutionary dynamics were more complex. Initially, cells produced
275 predominantly pyoverdine, such that only pyoverdine was exposed to selection while pyochelin was shielded from selection by the negative regulatory link (Fig. 3, left column). In time, mutants defective for pyoverdine production arose and spread (Fig 4), consistent with our understanding that pyoverdine is an exploitable public good under strong iron limitation (Fig. 2). Selection against pyoverdine in turn weakened its suppression of pyochelin, causing pyoverdine-negative mutants to begin producing
280 pyochelin. Accordingly, we saw that populations diversified into several distinct phenotypes, producing less pyoverdine and, in many cases, more pyochelin (Fig. 4). In our third selection regime – that of alternating exposure to strong and moderate iron limitation – the phenotypic slide towards lower siderophore production was qualitatively similar to, though less pronounced than, that seen under constant selection (Fig. 3). This result is consistent with the view that a fluctuating environment

285 can, in moderation, reduce selection for cheats (Brockhurst et al., 2007) and instead favour generalists (i.e. an individual-level solution; e.g. Kassen, 2002, Dumas et al., 2013) or stabilise phenotypic polymorphism (i.e. a lineage- or population-level solution; Kassen, 2002, Venail et al., 2011).

Although the population-level evolutionary dynamics largely followed the predicted patterns (Fig. 1), 290 the level of interclonal variation in siderophore phenotypes that emerged under strong iron limitation was somewhat surprising (Fig. 4). For instance, why did pyoverdine-negative mutants not spread to fixation, and why did we not see the rise of "double cheat" pyoverdine- and pyochelin-defective mutants as we had expected? After all, defined variants of such mutants have previously been shown to outcompete the wildtype across a range of conditions (Ross-Gillespie et al., 2007, Kümmerli et al., 295 2009b, Jiricny et al., 2010). We can posit a number of possible explanations, and they are neither exhaustive nor mutually exclusive. First, it may be that some of the isolates we assayed indeed carried mutations reducing expression of both siderophores, yet such mutants showed little net change in pyochelin production because their reduced production potential (owing to mutations in pyochelin genes) was masked by a concomitant boost in production effort (because mutations reducing 300 pyoverdine derepress pyochelin; (Dumas et al., 2013). Second, it may be that we simply did not continue the experimental evolution for long enough to see pyoverdine cheats fix and/or double mutants rise and spread to detectable frequencies. This seems plausible in light of a recent study which demonstrated that when bacteria are forced to adapt simultaneously to changes in both their abiotic and social environment, the rise and spread of cheats is relatively slower (Morgan et al., 2012). In other 305 words, what we see in our evolved lines could represent a transient situation, rather than some sort of stable equilibrium. Third, it may be that populations have indeed reached equilibria whereby multiple siderophore strategies can stably co-exist. Such coexistence could be facilitated by negative frequency-dependent selection, whereby the fitness of cheats decreases as they become more common, and eventually drops below that of the wildtype at some threshold frequency. Such dynamics have been 310 previously inferred from short term invasion experiments using this same model system (Ross-Gillespie et al., 2007) and are known to occur with microbial social traits too (Kerr et al., 2002, Ross-

Gillespie et al., 2007, Jousset et al., 2009, Barrett et al., 2011, Raymond et al., 2012). Finally, co-existence of different social strategies could also potentially come about through mutualistic interactions between community members (MacLean et al., 2010, Driscoll et al., 2011). This scenario would involve a division of labour whereby strains reciprocally swap different public goods at the population level. While mutualism could – in principal at least – evolve in the long run, for it to be stable, it would need to be accompanied by the coevolution of mechanism(s) to repress competition and keep cheats in check (Frank, 2003).

Whether stable or transient, it is nonetheless interesting to reflect on why we see greater phenotypic diversity in lines selected under strong iron limitation as compared to those selected under moderate iron limitation. First, let's consider the relative strength of selection. Moderate iron limitation imposes weaker selection and permits the persistence of larger populations, which can shelter a diversity of marginally maladapted phenotypes – variants that can serve as a backdrop for further mutations. Consequently, under this regime we might expect evolution to ultimately arrive at sophisticated phenotypes that are close to theoretical optima (Weissman et al., 2009, de Visser & Krug, 2014). Under strong iron limitation, meanwhile, conditions are harsher, population size is lower, and fewer variants can survive concurrently. Consequently, evolution could proceed more erratically, and may be more likely to converge on proximate, rather than ultimate, fitness optima (de Visser & Krug, 2014). Such a scenario could generate discrete clusters, rather than a cloud of phenotypes. Second, differences in the genetic architecture underlying the two traits could potentially impose different constraints on the evolutionary routes available to each trait (Koonin & Wolf, 2010), and thus could lead to different patterns of diversification. However, we consider this unlikely to matter much in the present case, because the genetic architecture underlying pyoverdine and pyochelin is not strikingly different (Visca et al., 2007, Youard et al., 2011): in both cases, synthesis is effected by non-ribosomal peptide synthetases and is controlled by a single major regulator (PvdS, in the case of pyoverdine, and PchR, in the case of pyochelin). Moreover, targeted mutagenesis of these regulators often leads to incremental reduction in siderophore investment (Michel et al., 2005, Wilson & Lamont, 2006). Most

of the altered phenotypes we observe are incremental, and therefore probably caused by changes in these two functionally analogous genes.

Taken together, our results support the view that pleiotropy caused by regulatory cross-links between traits can be an important modulator of the evolutionary dynamics of cooperative traits in microbes. Indeed, some types of regulatory links may have explicitly evolved because of their stabilizing effects on cooperation (Foster et al., 2004). As an example, let's consider quorum sensing (QS) (Williams et al., 2007), a form of cell-to-cell communication that facilitates synchronised expression of costly cooperative traits only when it is most beneficial to do so – at high cell density (Darch et al., 2012, Ross-Gillespie & Kümmerli, 2014). Cheating mutants – that do not respond to other cells' QS signals yet still benefit from the QS-controlled public goods these others produce – have repeatedly been observed to arise and spread (Sandoz et al., 2007, Rumbaugh et al., 2009, Wilder et al., 2011). However, since QS co-regulates many traits (i.e. an example of positive hierarchical regulation – see Fig. 1a), it may be that in some settings, cheats that would otherwise benefit by avoiding investment in one trait are kept in check by costs arising from the pleiotropic down-regulation of another trait. Such pleiotropic costs can include, for example, increased vulnerability to protist grazers (Jousset et al., 2009) or toxins (Jousset et al., 2013), or reduced metabolic capability (Dandekar et al., 2012). In our system, the negative regulatory linkage between pyoverdine and pyochelin fine-tunes the siderophore profile to prevailing environmental conditions (Dumas et al., 2013), and has probably evolved primarily for this reason. However, here too linkage may help to stabilize cooperation: because pyoverdine-defective cheats pleiotropically become pyochelin-producing cooperators, their relative advantage is reduced and their spread may thus be hindered.

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Figures and figure legends

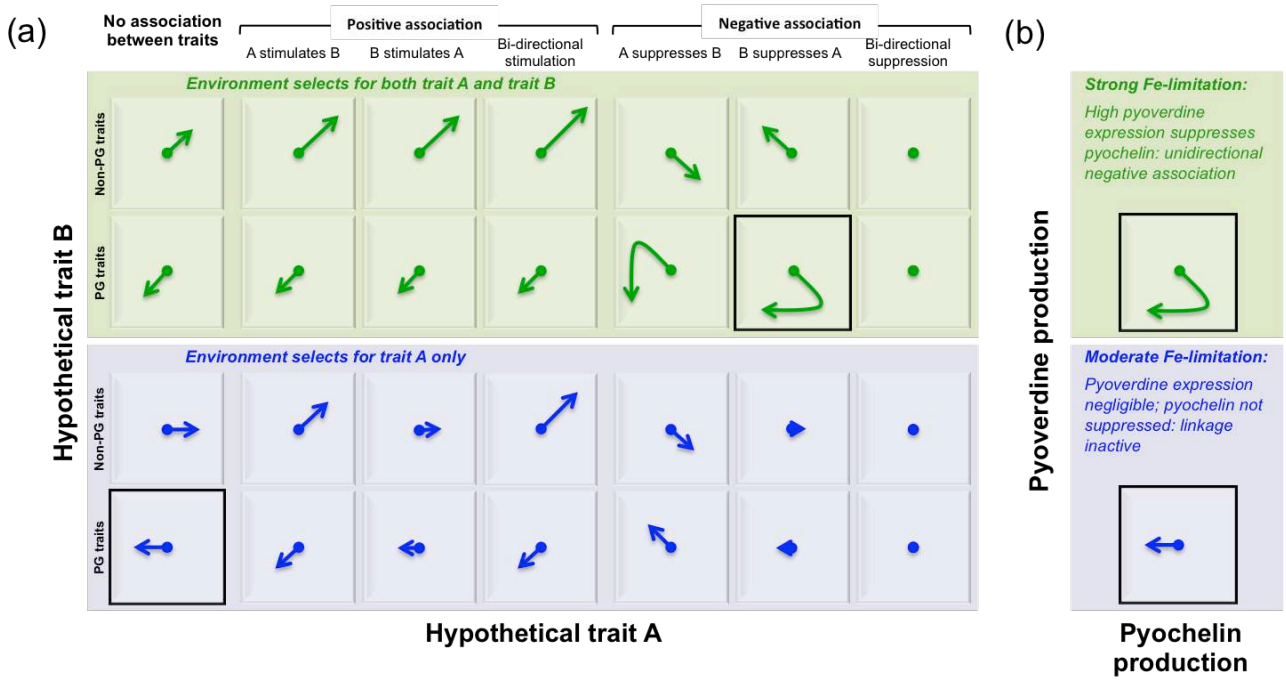

Figure 1. Predicted evolutionary trajectories for two interacting public goods. Part (a) sets out general predictions for hypothetical cases where two traits are, or are not, public goods (rows) and where the traits do, or do not, influence the level of expression of the other (columns), in environments that select for both (upper green block), or just one of the two traits (i.e. trait A; lower blue block). Part (b) transcribes these general predictions to the specific case of *P. aeruginosa*'s investment into pyoverdine vs. pyochelin, when faced with high vs. moderate iron limitation. Briefly, the logic underlying part (a) is as follows. We assume that phenotypes (i) are initially expressed at some low baseline level, (ii) change gradually, (iii) cannot be completely lost (i.e. no purging of disused genes), and can (iv) evolve freely, without constraints from, for example, limited mutation supply, metabolic trade-offs or the physical complexities of genome architecture. Moreover, we assume (v) no modification of the linkage patterns themselves. Under these assumptions, we would expect evolution to lead to increased investment into traits that are adaptive under prevailing conditions (top row of upper and lower blocks). If two traits are linked positively (columns 1-4), this could accelerate their augmentation (upper block), or even allow non-adaptive traits to hitchhike on selection on the focal trait (lower block). Negative linkage, meanwhile, when unidirectional, could mean one trait is necessarily traded off against the other, and when bidirectional, could block expression altogether and hence preclude any selective changes. With public goods traits, the expected patterns are quite different (lower rows of either block). Here, the rise of cheats within a population could skew evolution towards decreased investment into the focal trait, even in environments where the trait would ostensibly be beneficial. Under this scenario, positive linkage could cause pleiotropic declines in non-focal traits, while a negative association could see declines in focal traits leading to associated increases in non-focal traits. Where the environment acts on both traits (upper block), we could even see biphasic sequential dynamics – whereby an invasion of cheats that drags down investment in one trait is followed by a second invasion that then decreases investment in the second trait.

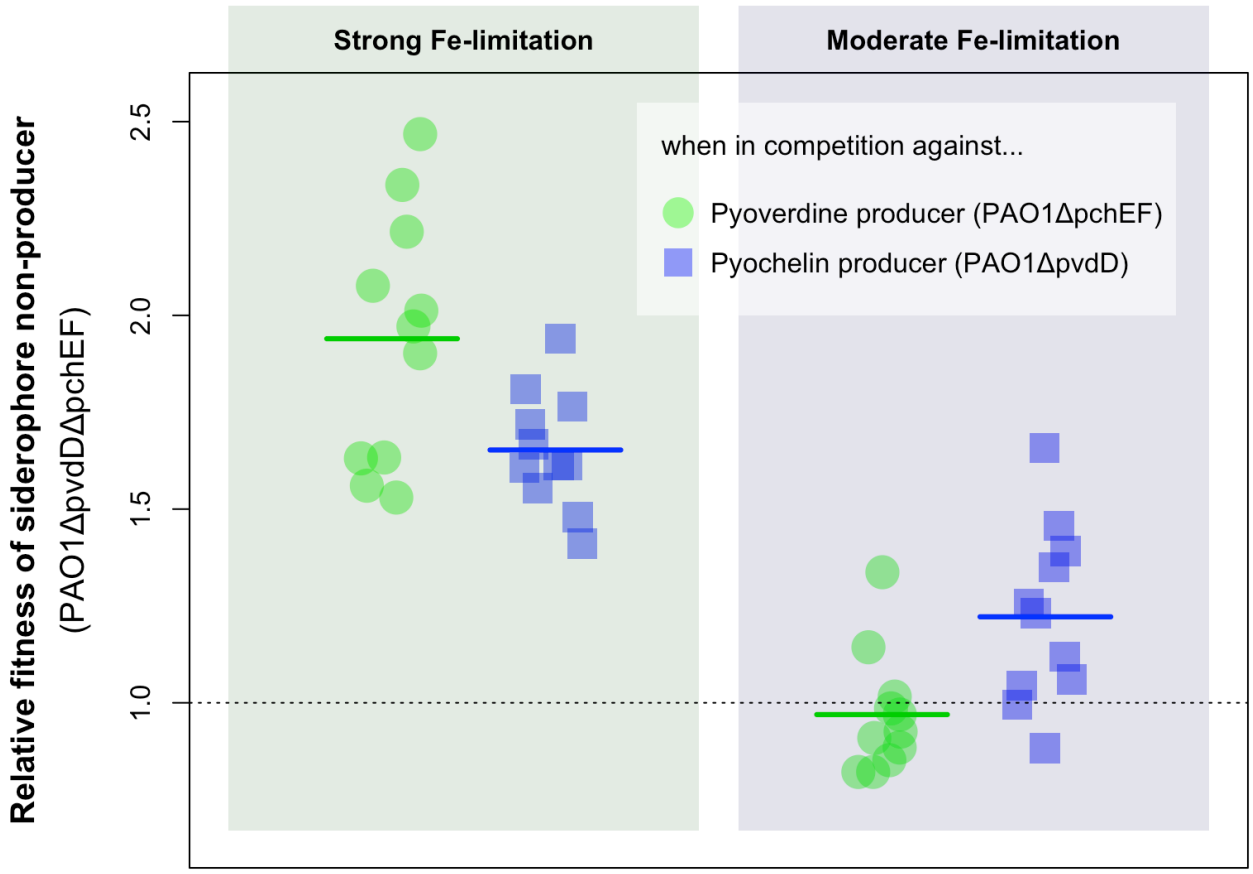

Figure 2. Siderophore non-producers can exploit and invade producers, but their relative advantage is environment-dependent. Symbols denote independent replicates and lines denote means within treatments. Relative fitness values greater than 1 (dotted line) indicate cases where the relative proportion of non-producers in the population increased during competition.

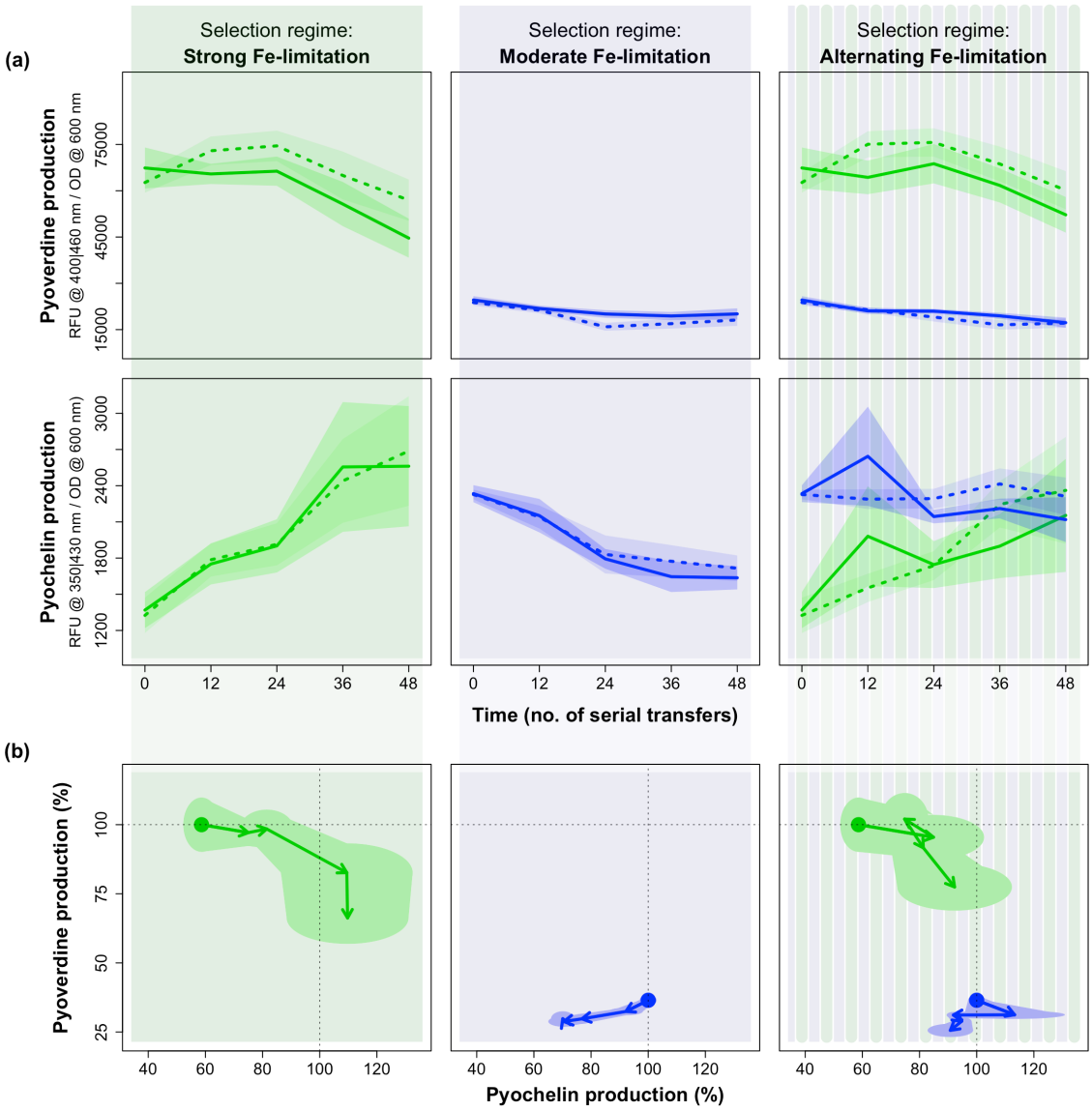

Figure 3. Changes in siderophore phenotype during experimental evolution under different iron limitation regimes. Evolving populations were assayed at transfers 0, 12, 24, 36 and 48, using medium that was strongly (green) or moderately (blue) iron-limited, matching the conditions experienced during selection. In (a), siderophore investment is shown as *per capita* fluorescence at siderophore-specific wavelengths, while in (b), these measures are further scaled relative to the ancestor, assaying under strong (for pyoverdine) or moderate (for pyochelin) Fe-limitation respectively. Solid lines track means at a clonal level (i.e. random samples of 10 individually-assayed isolates from each of n = 8 selection lines), while the dotted lines in part (a) represent measures taken at the population level (i.e. selection line). Shaded areas denote standard errors around the means.

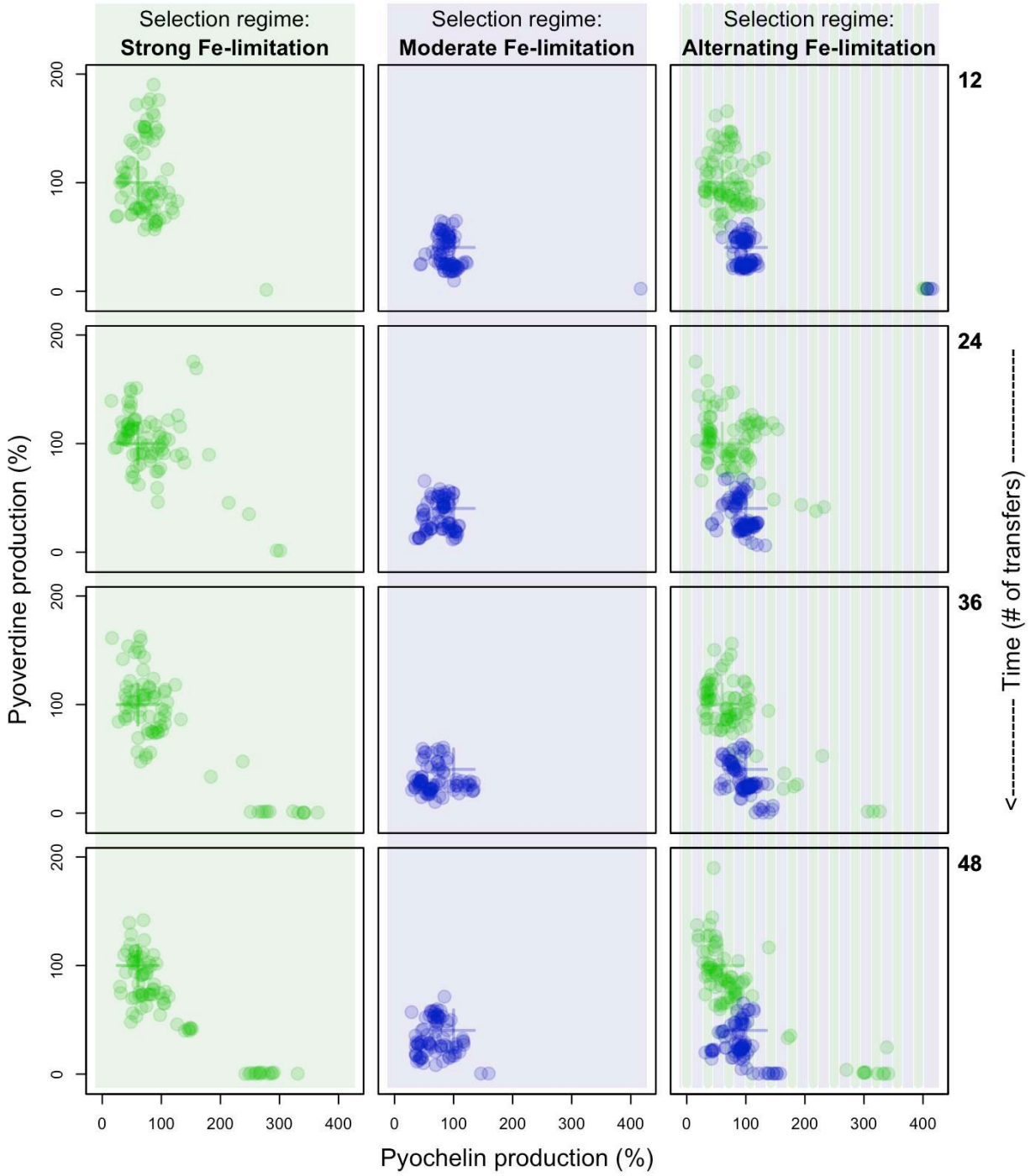

Figure 4. Intraclonal diversity in siderophore phenotype during experimental evolution under different iron limitation regimes. Random isolates (round symbols; n = 10) from each of 8 lines were assayed in medium that was strongly (green) or moderately (blue) iron-limited, matching the conditions experienced during selection. Optical measures of siderophore production measures are scaled as per Fig 3b. – i.e. relative to the ancestor's production under the same test conditions (i.e. 100%; shown here as crosses).

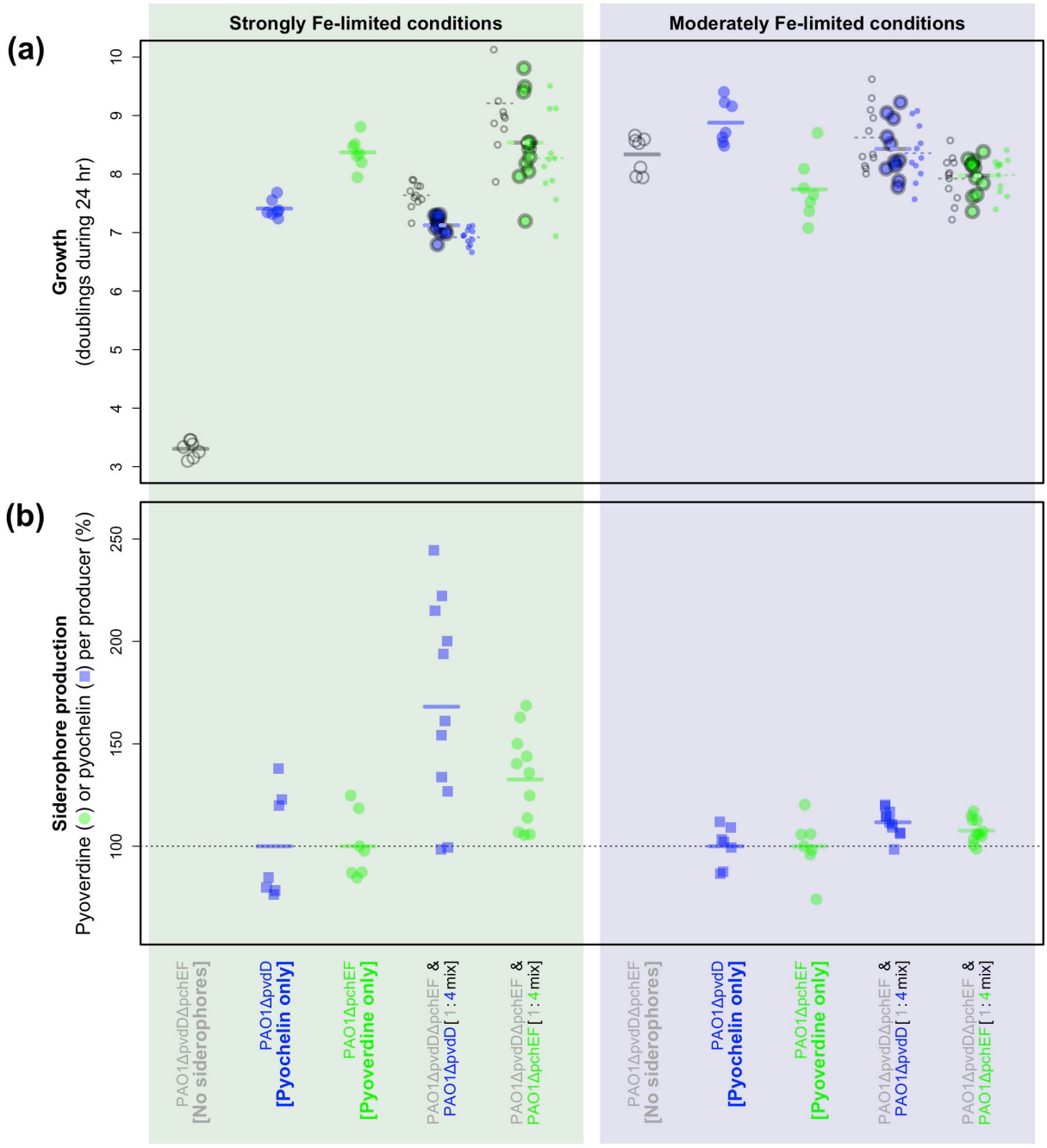

Figure S1. Both pyoverdine and pyochelin can act as public goods under appropriate conditions. (a) Growth (number of doublings during 24 hours, assuming exponential growth) is shown under strong and moderate Fe-limited conditions, for both whole populations and, in case of mixed cultures, for subpopulations of respective strain types. Growth estimates for subpopulations are based on changes in in relative frequency from start to end of the observation period. Symbols denote independent replicates and lines denote means within treatments. In co-culture under strongly Fe-limited conditions, the siderophore non-producer, PAO1*ΔpvdDΔpchEF*, outgrew both the pyochelin-only producer, PAO1*ΔpvdD*, and the pyoverdine-only producer, PAO1*ΔpchEF* (paired, 2-tailed t-tests: $t_{10} = 18.17$, $P < 0.001$ and $t_{10} = 12.91$, $P < 0.001$ respectively), while under moderately Fe-limited conditions it outgrew only PAO1*ΔpvdD* ($t_{10} = 3.28$, $P = 0.008$) and not PAO1*ΔpchEF* ($t_{10} = -0.93$, $P = 0.377$). (b) Siderophore levels, assayed by fluorimetry (Relative fluorescence at 400|460 nm for pyoverdine; 350|430 nm for pyochelin), were divided by the producers' share of total cell density (OD at 600 nm), and then scaled relative to the relevant monoculture within each environment.

Compared to monoculture conditions, growth in co-culture with the non-producer PAO1*ΔpvdDΔpchEF* was generally associated with elevated siderophore output on the part of producers (ANOVAs: PAO1*ΔpvdD* $F_{1,16}$ = 10.80, P = 0.005 and $F_{1,16}$ = 9.40, P = 0.007 for strong and weak Fe-limitation, respectively; PAO1*ΔpchEF* under strong Fe-limitation $F_{1,16}$ = 10.72, P = 0.005). However, in the case of PAO1*ΔpchEF* growing under moderate Fe-limited conditions, this increase was non-significant ($F_{1,16}$ = 2.62, P = 0.125).